UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	: Linda H. Malkas
By the Examiner	: Huff, Sheela Jitendra
Docket No.	: 80371/5
Serial No.	: 10/083,576
Filed	: February 27, 2002
Group Art Unit	: 1643
Title	: METHOD FOR PURIFYING CANCER-SPECIFIC PROLIFERATING CELL NUCLEAR ANTIGEN

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. §1.132

Dear Sir:

I, Lauren Schnaper, declare as follows:

1. I am a co-inventor, along with Linda H. Malkas, Robert J. Hickey, Pamela E. Bechtel, Min Park, Derek J. Hoelz, and Dragana Tomic, of the subject matter disclosed and claimed in the United States Patent Application Serial No. 10/083,576, filed February 27, 2002, entitled METHOD FOR PURIFYING CANCER SPECIFIC PROLIFERATING CELL NUCLEAR ANTIGEN.

2. I am a co-author of an article entitled *Detection of the Cancer Specific Form of PCNA by Elisa Assay*, Proceedings of the American Association For Cancer Research, Abstract No. 2507, vol. 42, p. 466 (EXHIBIT A) and am a co-inventor of the subject matter which is disclosed in this publication and disclosed and claimed in the above-referenced patent application.

3. In addition to myself, Robert J. Hickey, Linda H. Malkas, Derek J. Hoelz, and Dragana Tomic are co-inventors of the above-referenced patent application and co-authors of the above-referenced article.

4. P. Wills and C. Lankford are named as co-authors in the above-referenced article but are not inventors of the subject matter described in the above-referenced article or in the above-referenced patent application, but were listed as co-authors of the above-referenced article in order to receive credit for having collaborated in the research program by contributing to the program by providing materials.

5. P. Wills (Phil Wills) provided purified XPG-GST fusion protein for the ELISA.

6. C. Lankford (Carla Lankford) provided MCF7 (cancer) and MCF10A (Normal) cell extracts for the ELISA.

7. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

DATE: 8/7/06

Lauren A Schnaper

Lauren Schnaper

EXHIBIT A

1. *Detection of the Cancer Specific Form of PCNA by Elisa Assay*, Proceedings of the American Association For Cancer Research, Abstract No. 2507, vol. 42, p. 466.

O^6-alkylguanine-DNA alkyltransferase (AGT). In vitro studies demonstrated that NNKOAc either directly, or as a result of DNA adduct formation, interferes with the repair O^6-mG by AGT. In this study, we show that NNKOAc depletes AGT in A/J mouse lung. We then compared the ability of NNKOAc and O^6-benzylguanine (O^6-bzG) to enhance the tumorigenic activity of AMMN in A/J mouse lungs. O^6-bzG is an established in vivo inhibitor of AGT. NNKOAc and O^6-bzG had similar effects on the levels of AMMN-derived O^6-mG at 4 and 96 h post-injection. NNKOAc and O^6-bzG enhanced the lung tumorigenic activity of a 0.75 μmol dose of AMMN to a similar extent. These data are consistent with the hypothesis that the pyridyloxobutylation pathway contributes to the lung tumorigenic activity of NNK in A/J mice by interfering with O^6-mG repair. To determine if AGT substrate pyridyloxobutyl adducts are present in lung DNA from NNK-treated mice, we measured the levels of O^6-[4-oxo-4-(3-pyridyl)-butyl]guanine(O^6-pobG) in lung and liver DNA 24 h after exposure to 10 μmol [5-^{3}H]NNK. This adduct was detected in liver but not lung DNA. The limits of detection were approximately 0.5 pmol O^6-pobG/μmol guanine. The implications of these findings will be discussed [Supported by CA-59887].

#2507 Detection of the Cancer Specific Form of PCNA by Elisa Assay. D. Tomic, D. J. Hoelz, P. Wills, R. J. Hickey, L. Schnaper, C. Lankford, and L. H. Malkas. *Greater Baltimore Medical Center, Towson, MD, and University of Maryland, Baltimore, MD.*

Proliferating cell nuclear antigen (PCNA) is a 36kD nuclear protein which is required for DNA replication (processivity factor of DNA polymerase δ) and DNA repair. Previously, using 2D-IEF-PAGE analyses, our laboratory discovered that malignant breast cells express a unique, acidic form of PCNA protein which can clearly be distinguished from the basic form of this protein found in non-malignant cells. Our research suggests that the acidic form of PCNA is, most likely, the result of a post translational modification. This finding is important because this unique form of PCNA in breast cancer cells could potentially serve as a powerful marker for the detection of this malignancy. Therefore, the purpose of this study was to develop an ELISA test, which can distinguish the malignant form of PCNA from the non-malignant form. We tested the hypothesis that xeroderma pigmentosum (XP)G protein, a structure-specific repair endonuclease similar to FEN1, and used in the nucleotide excision repair pathway is capable of distinguishing two forms of PCNA through binding affinities. To test this hypothesis, the protein isolated from the non-malignant breast cell line (MCF10A) and the breast cancer cell line (MCF-7) were used to measure the binding affinity of XPG to the acidic and basic form of PCNA in a modified ELISA assay. Standard curves, representing the correlation between absorbance and the abundance of the malignant and non-malignant form of PCNA were prepared and compared to each other. Serial dilutions of PCNA were tested in duplicate and the mean value of absorbance was calculated and used for comparison. Our results indicate that XPG protein has a different binding affinity for the malignant and non-malignant forms of PCNA. These results are the first to demonstrate that these two forms of PCNA can be distinguished by an ELISA assay that can be used clinically for the early detection of breast cancer.

#2508 Effects of Zinc Occupancy on the Function of Human O^6-Alkylguanine-DNA Alkyltransferase (AGT). Joseph J. Rasimas, Sreenivas Kanugula, Michael G. Fried, and Anthony E. Pegg. *Penn State College of Medicine, Hershey, PA.*

AGT is a small monomeric DNA repair protein whose homologs are found in a wide variety of prokaryotic and eukaryotic organisms. It is responsible for the repair of potentially mutagenic and cytotoxic alkyl and haloalkyl adducts of DNA, specifically at the O^6-position of guanine and, to a lesser degree, at the O^4-position of thymine. Unlike many proteins which are responsible for maintenance of genomic integrity, AGT is not an enzyme, but instead, restores DNA by irreversible transfer of adduct substituents to an internal active site sulfur atom (Cys145). Two crystal structures of human AGT (hAGT) have recently been published, and while both lend similar insight into the biophysical nature of this repair mechanism, they differ on one specific aspect of the protein's structure. One model suggests the presence of a zinc atom bound within a coordination sphere of at least four amino acid residues (Cys5, Cys24, His29, and His85) near the N-terminus, while the other model shows these residues in similar orientation, but lacking the transition metal ion. We have, therefore, begun to examine the structural and functional consequences of the relative occupancy of the protein's putative zinc binding site. In bacterial expression systems, recombinant hAGT is produced in increasingly larger quantities when growth media are supplemented with $ZnCl_2$ up to a concentration of 0.1 mM. Furthermore, metal-enriched hAGT samples with a molar zinc:protein binding ratio of 1.83 :1 (assessed by ICP-MS) demonstrate a 60-fold increase in repair rate constant over metal-stripped hAGT, as well as a 5-fold increase over conventionally purified protein samples with a ratio of 0.66:1. In addition, mutants of Cys5 and Cys24 (two of the putative zinc-binding residues) show 89% and 56% decreases in zinc occupancy compared to wild-type protein and repair methylated DNA substrate with activities of 17-fold and 3.5-fold less than wild-type AGT, respectively. Mutations and metal content manipulations have little or no effect upon the CD spectrum of hAGT proteins, suggesting that the overall structural fold of the protein is not modulated by the relative occupancy of the zinc site. Using an electrophoretic mobility shift assay with 16-mer oligonucleotides, differentially zinc-treated hAGTs and metal-binding residue mutants (C5A and C24A) also show the same affinity for binding to DNA. Repair deficient active s
occupancy show similar binding
ylguanine. We conclude, therefore, that while zinc is neither essential for DN repair by hAGT nor required for maintaining a functional fold of the protein, th presence of the transition metal ion bound within the polypeptide structu confers a mechanistic enhancement to repair activity which does not result fro an increase in substrate binding affinity. Zinc may also provide some measure structural stability to hAGT.

#2509 Molecular Alterations in the Transcription-Coupled Nucleotide Excision Repair Gene, CS-B/ERCC6, in Human Malignant Gliomas. Franc Ali-Osman, Kurt Jaeckle, Thomas Connor, Gamil Antoun, and Lixin Zhan *U.T. M.D. Anderson Cancer Center, Houston, TX.*

The CS-B/ERCC6 gene encodes a complementation factor required for effi cient transcription-coupled nucleotide excision repair (TC-NER), a major DN repair pathway by which a variety of lesions are removed from the cellula genome. In this study, we examined 39 primary human malignant glioma spec mens and their matched normal tissues, as well as, 11 early passage glioma ce lines, for molecular alterations (deletions and mutations) in the CS-B gene. Th results were correlated with the histological grade of the tumors. The result showed, overall, CS-B gene deletions to increase with increasing glioma grad and exon II to be most frequently deleted exon. Frequencies of exon II deletion were 12.5%, 30.8% and 66.7%, in astrocytomas, anaplastic astrocytomas an glioblastoma multiformes, respectively. Mutation analysis performed by SSC analysis of exon II and confirmed by nucleotide sequencing, showed a lowe frequency of mutations in exon II of the CS-B gene in the tumors, with only 12.5% of the glioma, all anaplastic astrocytoma or glioblastoma multiforme, to harbo any mutations. The mutations were varied and comprised of nucleotide trans tions of CAC(H)→TAC(Y) in codon 13 and CAA(C)→CGG(H) in codon 15, an transversions of TCT(S)→TAT(Y) and CAG(Q)→CAC(H) in codons 57 and 7 respectively. These data suggest that defective TC-NER, resulting from genet abnormalities in the CS-B gene, particularly, in exons 2 and 5, may contribute t malignant progression in gliomas.

#2510 Y-Box Binding Protein-1 Binds Preferentially to Single-Stranded Nucleic Acid and Exhibits 3' - 5' Exonuclease Activity. Hiroto Izumi, Toshihir Imamura, Gunji Nagatani, Tomoko Ise, Tadashi Murakami, Hidetaka Uramoto, Takayuki Torigoe, Hiroshi Ishiguchi, Yoichiro Yoshida, Minoru Nomoto, and Kimi toshi Kohno. *Department of Molecular Biology, University of Occupational and Environmental Health, Fukuoka, Japan, and University of Occupational and Environmental Health, Fukuoka, Japan.*

We previously have shown that YB-1 (Y-box binding protein-1) binds prefer entially to cisplatin-modified Y-box sequences. Based on structural and biochem ical data, we predicted that this protein binds single-stranded nucleic acids. In th present study we confirmed the prediction and also discovered some unexpected functional features of Y-box binding protein-1. We found that the cold-shoc domain of the protein is necessary but not sufficient for double-stranded DN binding while the C-tail domain interacts with both single-stranded DNA and RN independently of the cold-shock domain. In an in vitro translation systems, th C-tail domain of the protein inhibited translation but the cold-shock domain di not. Antibodies recognizing the protein showed a supershift when single-stranded oligonucleotides were used as a probe, but not when double-stranded oligonu cleotides were used. Both in vitro pull-down and in vivo coimmunoprecipitation assays revealed that Y-box binding protein-1 can form a homodimer. Deletion analysis mapped the C-tail domain of the protein as the region of homodimer ization. We also characterized an intrinsic 3' - 5' DNA exonuclease activity of th protein. The region between residues 51 and 205 of its 324-amino acid extent i required for full exonuclease activity. Our findings suggest that Y-box bindin protein-1 functions in regulating DNA/RNA transactions, and that these actions involve different domains and are influenced by dimerization of the protein.

#2511 Examining the Role of a Human 3'-5' Exonuclease (ExoN) In the Fidelity of DNA Polymerase α. Kevin R. Brown, Carole L. Galligan, and Violetta Skalski. *University of Toronto, Toronto, ON, Canada.*

3'-5' exonucleases are proteins that ensure the accuracy of DNA replication by catalyzing the removal of mispaired nucleotides from the 3'-termini of nascen DNA. Base mutations arising through nucleotide misinsertions are considered to be important contributors to the development of cancer. Of the polymerases thought to be involved in DNA replication, only DNA polymerase alpha (pol α) lacks an intrinsic proofreading exonuclease, although it has been suggested that an unassociated 3'-5' exonuclease may provide this function. We have purified a previously uncharacterized 3'-5' exonuclease (exoN) from the nuclei of either primary or established acute myeloblastic leukemia (AML), chronic lymphocytic leukemia (CLL), and human cervical tumor cells, as well as normal peripheral blood lymphocytes. The apparent ubiquitous nature of exoN suggests this exonuclease has a significant biological function. ExoN is a 46kDa monomer, and is active on single- and double-stranded DNA. Kinetic studies have shown that exoN binds very tightly to duplex DNA substrates, with a Km of 0.3nM (correctly base-paired 3'-ends). In this study, we have set out to examine the impact of exoN on the fidelity of the exonuclease-deficient mammalian DNA polymerase α. Using an in vitro fidelity assay, we have examined the removal of all 16 possible nucleotide pairs. ExoN was shown to efficiently remove mispaired nucleotides